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PRINCIPAL INVESTIGATOR: John A. Parrish, M.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital Corporation
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John A. Parrish, M.D.

E-Mail: lwatts@partners.org

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14. ABSTRACT

The Center for Integration of Medicine and Innovative Technology (CIMIT) is a consortium of nonprofit Boston-area institutions based at Massachusetts General Hospital and includes Brigham and Women's Hospital, Massachusetts Institute of Technology, Draper Laboratories, Beth Israel Deaconess Medical Center, Boston Medical Center, Boston University, Children's Hospital Boston, Harvard Medical School, Newton-Wellesley Hospital and VA Boston Healthcare System. The overall goal of CIMIT is to combine clinical and technological excellence and educational components to generate, develop, and reduce-to-practice innovative and high-impact concepts to improve military and civilian healthcare. CIMIT implements research programs in many clinical areas, with an emphasis on military medicine, supported by basic science and engineering development in biomaterials, endoscopic tools, energy delivery, medical imaging, and other novel technologies.

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CIMIT Annual Report for DAMD17-02-2-0006
October 1, 2008 to September 30, 2009

Optical Enhancement in Videofetoscopy

Dario Fauza, MD, Principal Investigator
CIMIT Award # 06-015
Quarter Ending September 30, 2009

Overall Goals and Approach

Current videofetoscopic techniques demand the replacement of the amniotic fluid with an optically neutral solution, such as Ringer lactate or normal saline, due to the limited light diffusion and poor visibility of the fetus through the amniotic fluid. Such replacement can lead to complications such as dissection of the gestational membranes, infection, and preterm labor. Originally, we proposed to initiate the development of a digital video system that would preclude the need for amniotic fluid exchange during videofetoscopy.

First, we had to measure the precise optical properties of the amniotic fluid at different gestational ages and thus be able to determine the optimal light wavelength for penetration through the fluid. It is well known that, in order to obtain maximum transmitted signal, an "impedance" adaptation of the complete optical system is needed between the light source, the receiving camera and the propagation medium – in this case, the amniotic fluid.

In a second phase of the study, digital image-based rendering manipulations were to be developed, in order to filter/enhance the video image in real time and hopefully eliminate the need for amniotic fluid exchange.

Progress This Past Quarter

We have completed the first phase of the project, namely the optical analysis of the amniotic fluid at different gestational ages, as detailed in a previous report. This was a study in and of itself, which was presented nationally before the "Third Annual Academic Surgical Congress", in Huntington Beach, CA. The formal title of the presentation was: "Steigman SA, Kunisaki SM, Wilkins-Haug L, Takoudes TC, Fauza DO. Optical properties of human amniotic fluid: implications for video fetoscopic surgery." A related original manuscript has been accepted for publication in the journal "*Fetal Diagnosis and Therapy*" – a premier publication in the field. As to any progress on the second phase of the project, please see below.

Issues of Concern

We have identified a suitable collaborator to conclude the second and final phase of this project, namely Dr. Gary Tearney at the Massachusetts General Hospital. More specifically, we will have access to at least one of Dr. Tearney's prototypes of a microendoscope fitted with spectrally-encoded endoscopy (SEE), which should allow us to test the hypothesis of performing videofetoscopy without the need for amniotic fluid exchange. Although this would not be image-based rendering as originally planned, it is arguably a more practical solution and the final goal would be the same, namely our ability to preclude the need for replacement of the amniotic fluid with crystalloid during a videofetoscopic procedure.

Patent Activity

Per the previous reports, a fairly extensive patent search on related instrumentation, performed by a patent attorney hired by the hospital's Technology Innovation and Development Office (TIDO), turned out negative as related to our project goals. This has led to a decision by the hospital to file for a provisional patent application of a generic device. The decision to file for a definitive patent application will depend on the results of the second phase of the project, as well as the existing intellectual property on SEE-fitted microendoscopes.

Label-free detection and quantification of circulating leukocytes by *in vivo* flow cytometry

Charles P. Lin, Principal Investigator
CIMIT Project # 09-445
Quarter Ending September 30, 2009

Overall Objectives and Approach

Currently, obtaining white blood cell counts in the clinic requires blood withdrawal and laboratory analysis of the sample. Blood cell counts are necessary to diagnose and monitor infections, inflammatory diseases (such as sepsis), and disease-related leukopenia or neutropenia. In many of these cases, more frequent monitoring of the white blood count (WBC) would be beneficial, but is often not clinically feasible. The proposed research aims to develop a two-photon *in vivo* flow cytometer (TIFC) that continuously and non-invasively monitors the WBC without the need for multiple blood withdrawals and laboratory analysis.

We postulate that an *in vivo* two-photon fluorescence flow cytometer can be used to identify and count cells based on their tryptophan fluorescence without the need for labeling with an external fluorophore, allowing non-invasive, *in vivo* enumeration of the circulating white blood cells. Specific aims include:

1. Construct a two-photon-based *in vivo* flow cytometer for the excitation and detection of tryptophan fluorescence (with 590 nm pulsed laser light)
2. Test and characterize the constructed system
3. Develop an *ex vivo* test of the instrument specific for detecting flowing leukocytes, using micro-fluidic flow channels
4. Measure the number of circulating leukocytes *in vivo* in a mouse, based on the tryptophan fluorescence, and confirm accuracy of these counts

If successful, the device developed will have the potential to allow constant monitoring of the WBC for clinical applications such as sepsis detection for newborns in the NICU, detection of infection in pediatric patients, and monitoring of leukocyte levels in cancer patients.

Progress on Specific Aims

<u>Aim 1: System design and construction</u>

The goal of aim 1 is to build an *in vivo* two-photon flow cytometer for simultaneous detection of two-photon excited tryptophan fluorescence and one-photon fluorescence of DiD. Aim 1 is completed. The two-photon *in vivo* flow cytometer (TIFC) setup has been constructed as described in the previous quarterly report.

Aim 2: Testing and characterization of the TIFC system

The goal of aim 2 is to test and characterize the system constructed in aim 1. A number of basic characterizations of the system have been performed. The total power throughput of the system has been measured as 45%. This is close to the expected value given the estimated losses of each optical component within the system.

The system noise has also been measured and reduced to a negligible amount. This was done by analyzing the signal detected by the two-photon fluorescence detector with no sample in the field of view as a function of various configurations of emission filters. The background intensity of the system was defined as the number of background pixel peaks, which had a grayscale value greater than 10 in the TIFC trace that was acquired by an 8-bit video snapper card, as described in the previous progress report. A threshold value of 10 was chosen due to an issue with the sync electronics used to control the image acquisition which limits the minimum grayscale value to 10 rather than to 0, currently. The electronics are currently being redesigned to correct this problem. The number of background pixels, with no sample in the focal plane, as a function of increasing excitation laser power is shown for a number of filter combinations in Figure 1. A corresponding increase in the background signal with increasing laser power indicated that the filter combination was not fully rejecting the scattering of the incident laser power. The three filter combinations that demonstrated the least amount of system noise were then used to acquire tryptophan fluorescence images from cultured multiple myeloma cancer cells sitting stationary in the focal plane of the TIFC (data not shown). The signal-to-background ratio was calculated for at least eighteen cells per emission filter combination. From this data, it was determined that the best signal-to-background ratio was achieved with the combination of emission filters including a BG-3 color glass filter from Edmund Optics and a Semrock 357 nm bandpass filter. This filter combination was then used for all future experiments.

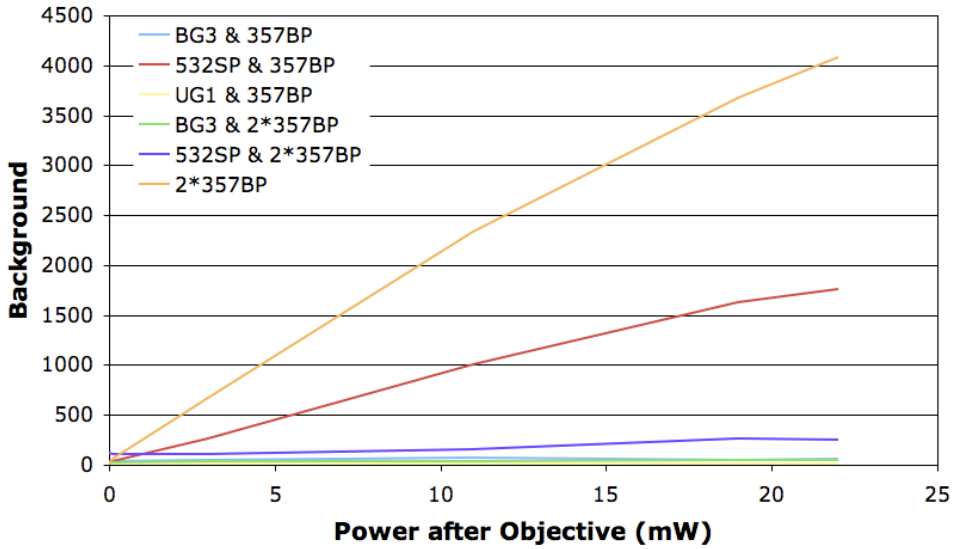

Figure 1. Plot of number of background pixel peaks as a function of the excitation laser power, as measured at the sample plane, for multiple emission filter combinations.

The tryptophan fluorescence intensity was also measured as a function of the numerical aperture (NA) of the objecti ve used in the system (to fo cus light on the sample and collect fluorescence from the sample). Th is was done by collec ting TIFC fluorescenc e data from cultured myeloma cancer cells sitting stationary in the foca l plane of the TIFC with different Olympus microscope objectives, including a 60x 1.20 NA water-immersion objective, a 60x 0.90 NA water-immersi on objective, and a 40 x 0.80 NA water-immersion objective. The average fluorescence intensity was calculated from images of at least eight cells per objective. The avera ge fluorescence intensity, normalized to the average fluorescence of the 1. 20 NA objective, is shown in F igure 2. The highest NA, 1.20, yielded the highest aver age fluoresc ence, as expected. Howev er, this objectiv e yields the smallest focal volu me in the sample plane, reduc ing the region of excitation within the sample. This volume of exc itation will need to be fu rther explored to determine what the best trade-off is between excitation volume and fluorescence signal intensity in order to achieve a high fluorescence signal whil e exciting as large a volum e within the blood vess el as pos sible, to increase the probabili ty of detecting as many cells flowing through the vessel as possible . This will be further exp lored as part of aim 3.

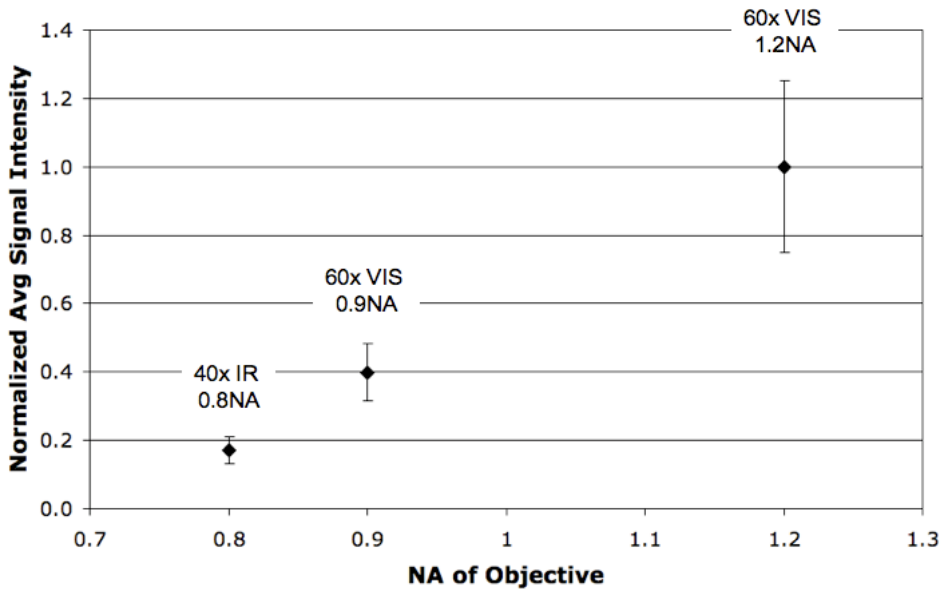

Figure 2. Plot of the normalized average fluore scence intensity of myeloma cells on a glass s lide using different microscope Olym pus objectives to focus the exc itation light and collect the fluorescence emission.

Since all previous c haracterizations hav e been done with cultured human multiple myeloma cancer cells, due to the ease of obtaining such cells from culture, the tryptophan fluorescence intensity was measur ed for various leukocyte populations and compared to the myeloma cells to better un derstand the fluorescence signal intensities expected from the leukocyte pop ulations of interest. Human leuk ocytes from a blood sample were sorted into two popula tions: lymphocytes (agranulocytes) and

granulocytes. Both populations of leukocyt es and the myeloma cells were imaged on a microscope slide under the same imaging conditions using a home-built, video-rate two-photon microscope with similar s canning and image acquisition as the TIFC. Example images ac quired of the stationa ry cells are shown in Figure 3a. In the image of the granulocytes (center), there appears to be two different populations of cells, which have not yet been identified. The fluorescence intensity of the cell populations was then determined and compared, as shown in F igure 3b. Cells in the granulocyt e population were analyzed as a single po pulation of cells as well as divided into two sub-populations: bright and dim. The tryptophan fluorescence fr om the bright granulocytes (yellow bar) was 1.9 times higher than that of the multiple myeloma cells (purple bar). The tryptophan fluorescence intensity of the dim granulocytes (green bar) was 60% that of the multiple myeloma cells, and the ly mphocytes yielded a fluorescence intensit y 63% that of the myeloma cells.

In future experiments, we need to determi ne what minimum fluorescence intensity is required to detect cells *in vivo*. Then, as we continue to use cultured myeloma cells for testing, we will b e able to use the comparis on data shown in F igure 3b to estimate the expected intensity of the leukocytes of inte rest. Th is information will allow us to estimate whether the leukocyte population of interest will be detectable *in vivo*.

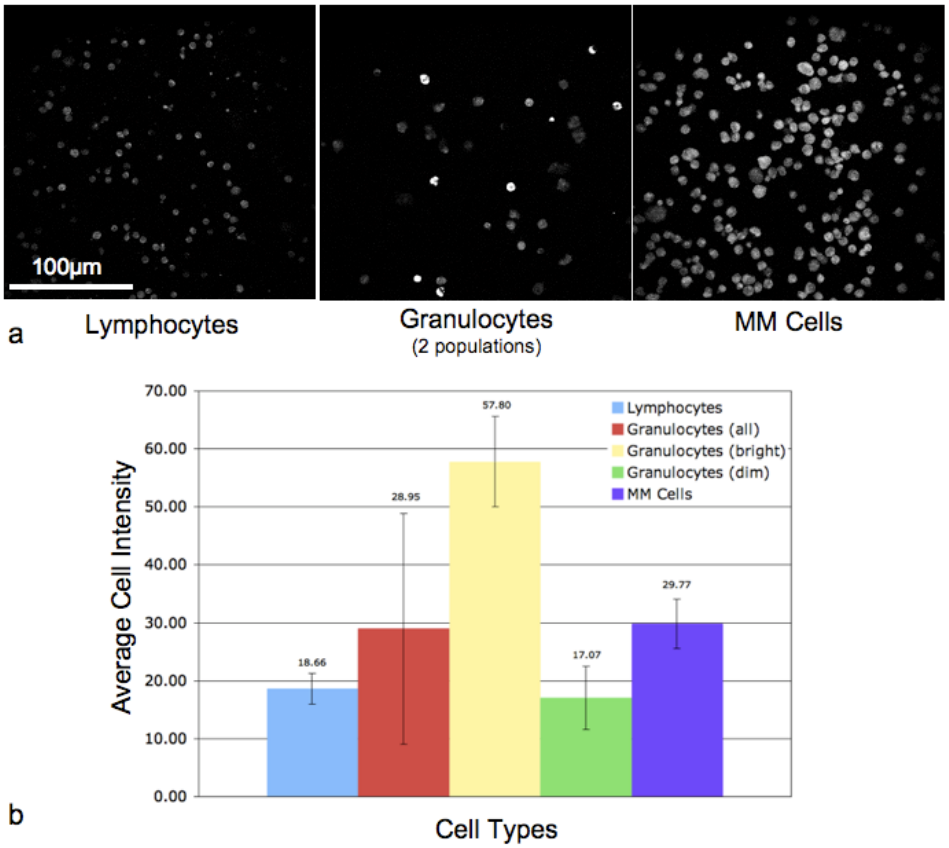

Figure 3. (a) Two-photon tryptophan fluorescenc e images from human lymphocytes (left), granulocytes (center), and cultured mult iple myeloma cancer cells (right). In the image of the granulocytes (cent er), there appears to be two different populations of

cells, which have not yet been identified. Scale bar is 100 μm. (b) Bar chart comparing the average tryptophan fluorescence intensity of each of the cell types.

Aim 3: Develop micro-fluidic set-up to test detection of cells *ex vivo*

Aim 3 will focus on *ex vivo* tests of the instrument built in aim 1. Micro-fluidic channels will be used as artificial blood vessels for these experiments. This aim will involve flowing cells through micro-channels and using tryptophan fluorescence to enumerate the cells, both in the absence and presence of hemoglobin. To accomplish this, a fluid pump is needed to move fluid through the micro-channels at physiological speeds. A Harvard Apparatus infusion pump that will yield flow speeds in the range of 0.15 mm/sec up to 3 m/sec for a 40 μm-diameter channel (similar in size to a blood vessel of interest) has been chosen as the best available pump for simulating blood flow *ex vivo*. This pump covers the possible speed of blood flow in a live mouse (1-10 mm/sec), which will allow us to test the detection of the TIFC at physiologically relevant speeds.

The pump needs to be ordered and the micro-channels set up, then experiments described under aim 3 can be conducted. These experiments will test the detection efficiency of the TIFC. The first portion of aim 3 will test the TIFC's ability to accurately detect leukocytes traveling at physiological speeds. The second portion of aim 3 will investigate the effect of blood (absorption by hemoglobin) on the detected tryptophan signal.

Aim 4: Measure the number of circulating leukocytes *in vivo*

Aim 4 will focus on detecting leukocytes circulating *in vivo* in a live mouse. In order to show that tryptophan fluorescence from leukocytes is detectable *in vivo*, cells in the vasculature and in the dermal layer of mouse skin were imaged, *in vivo*, using a video-rate two-photon microscope with the same excitation laser and image acquisition set-up and a similar scanning system as the TIFC. Two-photon tryptophan fluorescence images of rolling and arresting leukocytes were acquired *in vivo* in a live mouse. Figure 4a shows a rolling leukocyte (white arrow) in a blood vessel in the ear of a normal mouse. Figure 4b shows dermal cells (DC) in the tissue surrounding a blood vessel (bv) in a normal mouse. Figure 4c shows leukocytes (both bright, denoted by the yellow arrows, and dim, denoted by the green arrows) rolling and arresting in a blood vessel in the mouse ear 24 hours after injection of lipopolysacchride (LPS) to induce local inflammation. These images show that the tryptophan fluorescence yielded by leukocytes is detectable *in vivo* using two-photon excitation.

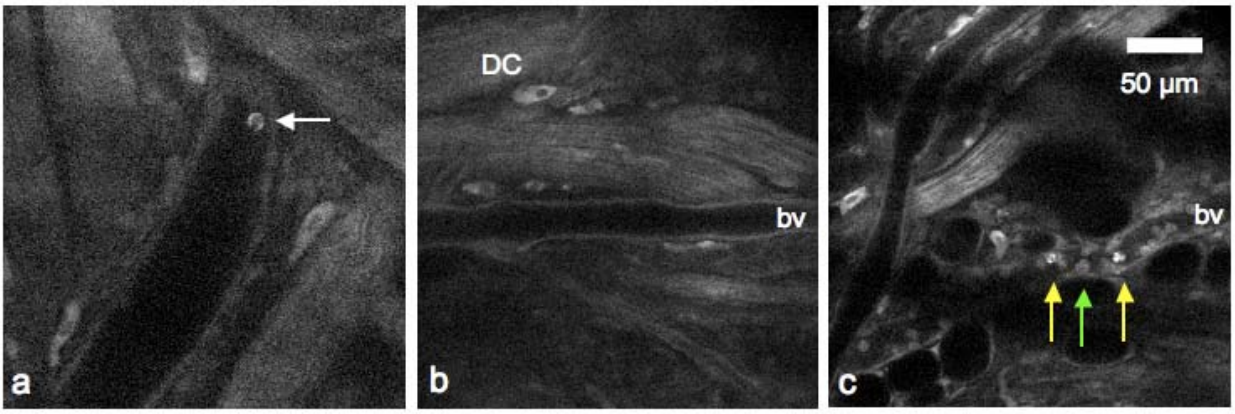

Figure 4. Two-photon tryptophan fluorescence images of rolling and arresting leukocytes acquired *in vivo* in a live mouse. Image (a) shows a rolling leukocyte (white arrow) in a blood vessel in the ear of a normal mouse. Image (b) shows dermal cells (DC) in the tissue surrounding a blood vessel (bv) in a normal mouse. Image (c) shows leukocytes (both bright, denoted by the yellow arrows, and dim, denoted by the green arrows) rolling and arresting in a blood vessel in the mouse ear 24 hours after injection of lipopolysacchride (LPS) to induce inflammation in the ear.

Summary of Results

To date, aim 1 has been completed, which consisted of designing and constructing a two-photon *in vivo* flow cytometer to excite and detect both two-photon tryptophan fluorescence and one-photon DiD fluorescence. Characterizations of the system have been performed in aim 2, showing low system noise and good power throughput. It has been demonstrated that tryptophan and DiD fluorescence from cultured multiple myeloma cells are detectable *in vitro* and the relationship between the intensity of the tryptophan fluorescence from human leukocytes and the myeloma cells has been determined *in vitro*. Towards aim 4, we have shown using two-photon fluorescence imaging that rolling and arresting leukocytes are detectable *in vivo* via their tryptophan fluorescence.

The next steps will be to show that tryptophan fluorescence is detectable *in vitro* at flow speeds equivalent to that expected *in vivo*, and to determine the signal-to-background ratio required to detect these cells *in vivo*.

Poly(Glycerol Sebacate) Film as a Barrier to Formation of Viscero-Parietal Peritoneal Adhesions

Cathryn Sundback, Principal Investigator
CIMIT Project # 09-246
Quarter Ending September 30, 2009

Overall Objectives and Approach

Peritoneal adhesions readily form in response to tissue injury in patients undergoing major abdominal or gynecologi c surg ery. Visceroparietal (VP) peritoneal adhesions often result in costly c omplications and diffic ult subsequent surgeries. Clinical adhesion prevention strategies are primarily adhesion barriers that separate damaged tissu e during early inf lammatory healing. All commercially available, FDA-approved ba rrier products suffer from sever al drawbacks including poor degr adability, variable effic acy, and difficult handling characteristics.

Poly(glycerol sebacat e) has the potential to be an effective adhesion formation barrier as it is a highly biocompatib le biodegradable elastomer with non-adhesive wetted sur face character. The objective s of this st udy ar e to evaluate the efficacy of PGS films for the prevent ion of VP peritoneal adhesions and to demonstrate the ease of laparoscopic PGS film placement. The specific aims include:

1. Optimize PGS film fabrication
2. Demonstrate the efficacy of PGS f ilms as a barrier to visceroparietal adhesion formation in a standard rat adhesion model.
3. Demonstrate minimally invas ive im plantation of PG S films in a large animal model.

This study represents a critical first st ep toward pot ential us e of PGS films in barrier product applications.

Summary of Results

We have completed Aim 1, optimizing PGS f ilm fabrication. In addition, we have developed an effective rat adhesion model and are c onducting a short term pilot study (3 wk) to verify the model and demonstrate the efficacy of PGS barrier films to prevent adhesion formation.

<u>Aim 1: Optimize PGS film fabric</u>

To create sheets of any required dimension (width, height, and thickness), a novel thin sheet PGS processing methodology was developed. An open window mold of silicone rubber was created and layered upon a sucrose coated silicon wafer bottom, creating a trough; the height of this mold determined the thickness of the final PGS sheet. Melted PGS prepolymer was deposited into the trough of the window mold (Figure 1), spread using a straight-edged razor until level with

the surrounding silicone rubber rails, and then crosslinked at 120^0 C for a pre-determined time. After curing, the sucrose-coated silicon wafer with attached PGS sheet was immersed in deionized water, dissolving the sucrose and allowing facile demolding of the PGS sheet.

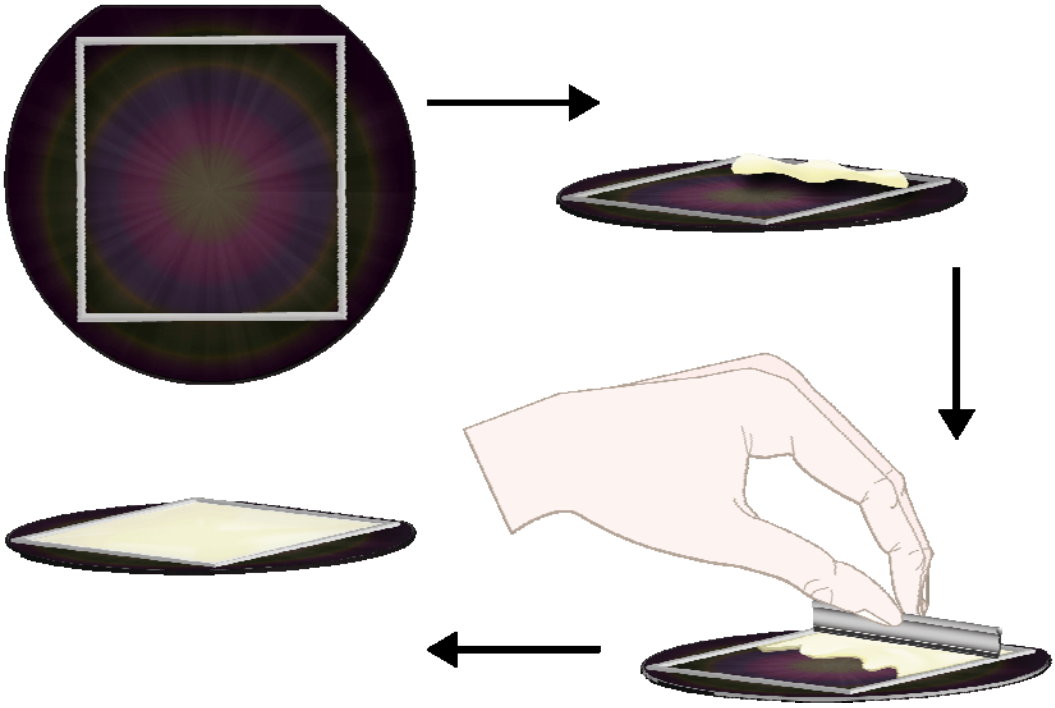

Figure 1: Creation of PGS sheets with silicone window

Aim 2. Demonstrate the efficacy of PG S films as a barrier to visceroparietal adhesion formation in a standard rat adhesion model.

A modified rat cecal abrasion and abdominal wall defect model was used as the peritoneal adhesion animal model. A 1 cm x 2 cm parietal peritoneal defect was created over the cecum by sharp abrasion, and the cecum was abraded until petechial bleeding was observed. In this initial model development study, a PGS film was placed between the abraded tissues for the "PGS implantation group" (n=3); control animals (n=3) underwent similar surgical procedures without PGS film placement. A larger subsequent trial will be conducted with 5 wk and 8 wk implantation times.

Publications and Presentations

Proposal Activities

Issues and Concerns
None